Allelism and molecular mapping of soybean necrotic root mutants

Reid G. Palmer, Lei Zhang, Zhiping Huang, and Min Xu

Abstract: Mutability of the *w4* flower color locus in soybean, *Glycine max* (L.) Merr., is conditioned by an allele designated *w4-m*. Germinal revertants recovered among self-pollinated progeny of mutable plants have been associated with the generation of necrotic root mutations, chlorophyll-deficiency mutations, and sterility mutations. A total of 24 necrotic root mutant lines were generated from a total of 24 independent reversion events at the *w4-m* locus. The initial mutable population included 4 mutable categories for *w4-m*, designated (1) low frequency of early excisions, (2) low frequency of late excisions, (3) high frequency of early excisions, and (4) high frequency of late excisions. These mutable categories were based upon flower phenotype, i.e., somatic tissue. A total of 22 of 24 necrotic root mutations occurred from germinal reversions classified in the high frequency of excision categories. Of these 22 mutants, 14 came from early excisions and 8 came from late excisions. These necrotic root mutants were allelic to 6 previously identified necrotic root mutants derived from the study of germinal revertants, i.e., gene tagging studies, chemical mutagenesis, and "spontaneous" occurrences from genetic crosses. Thus, all 30 necrotic root mutants in soybean are allelic. An F_2 mapping population from the cross of Minsoy (*Rn1 Rn1*) × T328 (*rn1 rn1*) was used to map the *Rn1* locus using simple sequence repeat (SSR) markers. The *Rn1* locus was located between Satt288 and Satt612 on molecular linkage group G.

Key words: disease lesion mimics, germinal revertants, molecular mapping, necrotic root, soybean.

Résumé : L'instabilité du locus *w4* déterminant la couleur des fleurs chez le soja (*Glycine max* (L.) Merr.) est déterminée par l'allèle *w4-m*. Des révertants germinaux obtenus au sein d'une progéniture autofécondée de plants portant l'allèle instable affichent diverses mutations produisant des lésions racinaires nécrotiques, une déficience chlorophylienne et la stérilité. Un total de 24 lignées mutantes à lésions racinaires ont été obtenues à partir de 24 réversions indépendantes au locus *w4-m*. La population initiale comprenait quatre types de mutations au locus *w4-m* : (1) des excisions précoces à faible fréquence, (2) des excisions tardives à faible fréquence, (3) des excisions précoces à haute fréquence et (4) des excisions tardives à haute fréquence. Ces catégories de mutations ont été établies sur la base du phénotype floral, c'est-à-dire observées sur un tissu somatique. Vingt-deux des 24 mutants à lésions nécrotiques ont été obtenus à partir de révertants germinaux appartenant aux catégories d'excisions à haute fréquence. De ces 22 mutants, 14 provenaient de mutants à excisions précoces et 8 huit provenaient de mutants à excisions tardives. Ces mutants à lésions nécrotiques étaient alléliques à six mutants à lésions nécrotiques identifiés précédemment au cours d'une étude portant sur des révertants germinaux issus de mutagenèse insertionnelle, de mutagenèse chimique ou issus 'spontanément' suite à des croisements. Ainsi, les 30 mutants à lésions nécrotiques chez le soja sont alléliques. Une population F_2 de cartographie génétique provenant du croisement Minsoy (*Rn1 Rn1*) × T328 (*rn1 rn1*) a été employée pour situer le locus *Rn1* à l'aide de marqueurs microsatellites (SSR). Le locus *Rn1* a été situé entre les marqueurs Satt288 et Satt612 sur le groupe de liaison G.

Mots-clés : mimétisme de lésions pathologiques, révertants germinaux, cartographie moléculaire, racine nécrotique, soja.

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Introduction

An unstable mutation for anthocyanin pigmentation in soybean, *Glycine max* (L.) Merr., was identified in 1983. The mutability is conditioned by an allele at the *W4* locus that is recessive to wild type (Groose et al. 1988). This is the *w4-m* allele and the mutant line was assigned Genetic Type Collection number T322 (Palmer et al. 1990). The mutable allele yields germinal revertants at a rate that varies from 5% to 10% per generation, and the revertant alleles are usually stable (Groose et al. 1990). Approximately 1% of the progenies derived from germinal revertant plants contain mutations at other loci (Palmer et al. 1989). The first gene tagging study (Palmer et al. 1989) generated mutants that included chlorophyll deficiency, root necrosis, female partial sterility, complete male and female sterility, and

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different flower color/distribution patterns. Thus far, no molecular evidence is available to confirm that the *w4* mutable phenotype in soybean is produced by transposable elements. Collectively, however, the data strongly suggest that a transposable element system is producing the unstable flower phenotype.

The second gene tagging study was initiated in 1988 (R.G. Palmer, unpublished data) and included the parents, X1878 and X2717, of the original line (Asgrow X25AF) that gave rise to *w4-m* (Weigelt et al. 1990) and cultivar Harosoy as a control (Weiss and Stevenson 1955). The 4 mutable categories, as visualized on flowers, for this gene tagging study included early (in floral ontogeny) reversions and low frequency of excision, early reversions and high frequency, late reversions and low frequency, and late reversions and high frequency. Few chlorophyll-deficient mutants were observed, but a large number of necrotic root mutants and sterility mutants were evident. A total of 24 independent germinal reversion events generated 24 necrotic root mutant lines and a total of 36 independent germinal reversion events generated 36 male-sterile, female-sterile mutant lines.

In the first gene tagging study, 3 independent necrotic root mutants were recovered (Palmer et al. 1989) and assigned Genetic Type Collection numbers T328H, T329H, and T330H (Kosslak et al. 1997). These mutants showed progressive browning of the root soon after germination that was associated with accumulation of phytoalexins and pathogenesis-related proteins (Kosslak et al. 1996). These mutant lines also showed an increased tolerance to root-borne infection by the fungal pathogen *Phytophthora sojae* (Kosslak et al. 1996).

Grafting and decapitation experiments indicated that the necrotic root phenotype was root autonomous (Kosslak et al. 1997). The presence of fragmented DNA, detected by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling), and electron microscopy observations suggested the occurrence of apoptosis. Therefore, the soybean necrotic root phenotypes represent programmed cell death mutants (Kosslak et al. 1997).

Two necrotic root mutations in soybean were considered spontaneous mutations (Palmer and Mahama 2005). Another necrotic root mutation was identified after seed chemical mutagenesis with ethyl methanesulfonate (EMS) (Palmer and Wubben 1998). These 3 mutant lines, and one of the 24 mutants from the second gene tagging study (ASR-6-45), have been tested for allelism with T328, T329, and T330, the previously characterized necrotic root mutants. All 7 mutant lines are allelic (Anderson and Palmer 1997; Palmer and Wubben 1998; Palmer and Mahama 2005).

The objectives of the present study were to (1) test the remaining 23 necrotic root mutant lines from the second gene tagging study for allelism and (2) molecularly map the necrotic root (*Rn1*) locus.

Materials and methods

Plant materials

Generation of mutants

Six mutable line designations representing 4 mutable categories were used in the second gene tagging study. Two mutable categories did not contain necrotic root plants among self-pollinated progeny of the germinal revertant plants. The 4 mutable categories, the number of germinal revertant entries, and the number and percentage of necrotic root phenotypes are given in Table 1. For comparison, the number and percentage of male-sterile, female-sterile phenotypes are also given in Table 1.

Allelism tests

Twenty-three of the 30 necrotic root mutants in soybean have not been tested for allelism. Genetic type T328 (*rn1 rn1*) was used as the standard (Kosslak et al. 1997) mutant for the allelism test with the unknown mutants.

Upon self-pollination of T328H (*Rn1 rn1*), normal plants (*Rn1 Rn1*) and necrotic root mutant plants (*rn1 rn1*) were observed in a ratio of 3:1. Single-wedge grafts were made using 8-day-old *rn1 rn1* plants as the scion and 10-day-old sibling *Rn1 Rn1* plants as the rootstock. The graft methodology followed the procedure of Albertsen et al. (1983). The wedge-shaped scion was inserted into the inverted-wedge shaped rootstock. Sterile water was applied to the graft union and lanolin was applied to minimize desiccation. A plastic drinking straw, previously cut lengthwise and on the rootstock, was moved upwards to encompass the graft union. The straw was held in place by rubber bands. A total of 33 successful grafts were made out of 38 attempts. These 33 grafted plants were used as male parents and crossed to normal plants (female parents) in families segregating normal and necrotic root phenotypes. A total of 326 F_1 (testcross) plants were generated.

The identity of each male and each female plant was maintained. Progeny testing of the 33 grafted male parents and the female parents in each of the 23 segregating families was done.

The 326 F_1 seeds were placed on germination paper in a growth chamber at 32 °C and the 8-day-old seedlings were classified as normal root or necrotic root. The normal root plants were saved and allowed to produce self-pollinated seed. A representative seed sample (F_2 seed) from each F_1 plant was placed on germination paper, and the resulting F_2 seedlings were classified for normal or necrotic root phenotype to confirm the heterozygosity of the F_1 plant.

Molecular mapping

Cultivar Minsoy (PI 27890) (*Rn1 Rn1*) was crossed with T328H (*Rn1 rn1*) plants to produce F_1 seeds. The F_1 plants were grown in the USDA greenhouse at Iowa State University and threshed individually. Sixteen seeds per F_2 family were germinated in a growth chamber at 32 °C, and the roots of seedlings were checked 8 days later to determine whether the F_2 family was segregating for necrotic root. One F_2 family that showed segregation of the necrotic root phenotype was chosen as the mapping population.

A total of 68 F_2 seeds randomly selected from one segregating F_2 family were germinated in the growth chamber at 32 °C to construct the population for mapping of the locus that conditions necrotic root. The 8-day-old seedlings with normal root were transplanted and grown in the field. As necrotic root mutants are usually lethal, the necrotic root seedlings were grafted onto healthy rootstocks from cultivar

Table 1. Generation of germinal revertant necrotic root and male-sterile, female-sterile phenotypes by mutable category, and number and percentage of mutant phenotypes per mutable category.

		Necrotic root			Male-sterile, female-sterile		
Mutable category	Mutable line designation	No. of entries	No. of phenotypes	% of phenotypes	No. of entries	No. of phenotypes	% of phenotypes
Low frequency, late excision	ASR-3	961	0	0	961	14	1.46
High frequency, early excision	ASR-6	410	14	3.41	410	0	0
Low frequency, early excision	ASR-7	236	1	0.42	236	1	0.42
Low frequency, early excision	ASR-8	294	0	0	294	0	0
Low frequency, early excision	ASR-9	747	1	0.13	747	2	0.27
High frequency, late excision	ASR-10	558	8	1.43	558	19	3.41
Total		1951*	24	1.23	2502*	36	1.44

Note: The mutable categories are based upon flower phenotype. Fifty flowers from each segregating entry of the 4 mutable categories were classified. Five flowers from 5 plants were collected on 10 July and 17 July 1988 from each of the segregating entries.

*Does not include entry ASR-3 (necrotic root phenotype) or entry ASR-6 (male-sterile, female-sterile phenotype).

BSR101 (Tachibana et al. 1987). The grafted mutants were grown in the USDA greenhouse at Iowa State University.

Each F_2 plant was threshed individually. Twenty-four $F_{2:3}$ descendants from each F_2 plant were germinated on paper to evaluate the genotype of the F_2 plant by checking root phenotype.

Molecular mapping with SSR markers

Genomic DNA from 68 F_2 plants and their parental lines was extracted from freeze-dried leaf tissue by the CTAB method (Keim et al. 1988).

Bulked segregant analysis (BSA) (Michelmore et al. 1991) was used to quickly identify SSR markers linked to the *rn1* gene. Based on $F_{2:3}$ phenotypic data, 2 bulks were established: bulk 1 (B1) was constructed by pooling DNA from 10 F_2 individuals identified as homozygous normal (non-necrotic) root (*Rn1 Rn1*), and bulk 2 (B2) was constructed by pooling DNA from 10 F_2 individuals identified as homozygous necrotic root (*rn1 rn1*).

SSR markers were amplified by the polymerase chain reaction (PCR) in a 15 μL reaction mix that contained 1× PCR buffer, 1.75 mmol/L $MgCl_2$, 0.15 mmol/L sense and antisense primers, 150 mmol/L each dATP, dCTP, dGTP, and dTTP, 50 ng of genomic DNA, and 2.5 U of *Taq* DNA polymerase (Promega). The PCR program employed was 32 cycles of 45 s at 94 °C, 45 s at 47 °C, and 45 s at 68 °C. PCR products were run on a 2.0% (*w*/*v*) Agarose 3:1 HRB™ (AMRESCO) gel in 0.5× TBE buffer.

Linkage analysis

The putative closely linked SSR markers identified in the BSA experiment were used to screen the whole F_2 mapping population. The recombination values between the markers and the *rn1* locus were estimated using the Linkage-1 computer program (Suiter et al. 1983).

Map positions of the *rn1* locus and the SSR markers in the final map were calculated with the program MAPMAKER 2.0 (Lander et al. 1987), using a minimum LOD of 3.0 and a maximum recombination value of 0.4 as thresholds. The genetic distances in centimorgans (cM) were calculated with the Kosambi mapping function (Kosambi 1944).

Results

Generation of mutants

In the second gene tagging study, parents X1878 and X2717 of the *w4* mutable line had 1227 and 996 entries, respectively, and no mutant phenotypes were observed. Cultivar Harosoy, also used as a control, had 1000 entries and no mutant phenotypes were noticed. Two mutable lines, ASR-3 (low frequency, late revertant events; 961 entries) and ASR-8 (low frequency, early revertant events; 294 entries), had no necrotic root mutant phenotypes (Table 1).

Only the lines in the high frequency categories, ASR-6 and ASR-10, generated necrotic root phenotypes with a frequency greater than 1%. The other lines in the low frequency, early excision category, ASR-7 and ASR-9, had frequencies less than 1% (Table 1).

Allelism tests

Progeny testing of the 33 grafted male parents gave all necrotic root progeny, which confirmed that necrotic root seedling selection and grafting were successful (data not shown). Progeny testing of the female parent in each of the 23 untested families identified the homozygous dominant genotype (*Rn1 Rn1*) and the heterozygous genotype (*Rn1 rn1*) (Table 2).

The allelism test crosses were confirmed heterozygous plants (*Rn1 rn1*) as female parent and known (grafted) homozygous recessive plants (*rn1 rn1*) as male parent. From 6 to 20 F_1 seeds were produced for each of the 23 cross combinations. F_1 plants were approximately equal numbers of normal and necrotic plants (Table 2). A representative seed sample from each normal F_1 plant was progeny-tested separately. A ratio of 3 normal plants to 1 necrotic root plant confirmed the heterozygous genotype of each normal F_1 plant (Table 2).

Molecular mapping

The mapping population consisted of 68 F_2 individuals randomly selected from one segregating F_2 family derived from a cross between Minsoy (*Rn1 Rn1*) and T328H (*Rn1 rn1*). The genotype of each individual in the mapping population was determined by progeny testing. The results showed that the mapping population consisted of 20 homo-

Table 2. Parents and F_1 and F_2 generations of allelism tests between T328H (*Rn1 rn1*) and 23 unknown soybean necrotic root mutants.

	No. of female plants			No. of F_1 (testcross) plants		No. of F_2 plants		
ASR entry No.	Normal	Necrotic	$\chi^2_{(3:1)}$	Normal	Necrotic	Normal	Necrotic	$\chi^2_{(3:1)}$
6-21	98	35	0.12	10	10	49	13	0.54
6-50	98	28	0.52	6	5	54	19	0.04
6-149	88	33	0.33	5	4	49	16	0.005
6-160	93	32	0.02	7	8	30	11	0.07
6-217	107	30	0.70	8	8	47	14	0.14
6-219	98	30	0.17	9	8	52	16	0.08
6-269	105	42	1.00	8	7	75	29	0.46
6-274	88	35	0.78	11	8	38	11	0.17
6-276	84	31	0.24	11	7	69	22	0.03
6-314	90	21	2.19	14	4	38	15	0.31
6-351	137	43	0.12	3	3	47	17	0.08
6-352	77	21	0.67	10	10	66	24	0.14
6-373	73	30	0.93	7	5	49	19	0.31
7-100	122	36	0.41	8	7	38	18	1.52
9-155	97	39	0.98	4	8	36	14	0.19
10-7	85	30	0.07	4	5	52	19	0.12
10-23	155	48	0.20	10	6	47	19	0.51
10-107	68	27	0.59	8	7	39	14	0.06
10-142	91	38	1.37	10	9	54	16	0.17
10-226	69	25	0.13	5	11	38	15	0.31
10-271	111	35	0.08	6	2	46	17	0.13
10-439	118	36	0.21	5	3	54	19	0.04
10-506	133	39	0.49	4	8	58	18	0.07

Note: ASR6-45 was previously tested for allelism (Anderson and Palmer 1997).

zygous wild-type individuals (*Rn1 Rn1*), 32 heterozygous individuals (*Rn1 rn1*), and 16 homozygous necrotic root mutants (*rn1 rn1*). The genotypic segregation ratio fit the expected 1:2:1 ratio ($\chi^2 = 0.7$; $P = 0.7$).

In the BSA experiment, 2 bulks (B1 and B2) and the parental lines were initially screened with 114 SSR markers polymorphic between Minsoy (*Rn1 Rn1*) and T328H (*Rn1 rn1*), which were identified in previous studies that represented all 20 linkage groups of the soybean genome (Song et al. 2004). Marker Satt288 on molecular linkage group (MLG) G detected polymorphism between the two bulks and between the two parental lines. The band amplified from B1 (*Rn1 Rn1*) by Satt288 had the same size as the one from Minsoy (*Rn1 Rn1*), and the band amplified from B2 (*rn1 rn1*) had the same size as the one from T328H (*Rn1 rn1*) (Fig. 1). According to the BSA strategy, Satt288 was a candidate marker linked to the *rn1* locus.

To confirm that Satt288 was closely linked to the *rn1* locus, Satt288 was used to screen all 68 F_2 individuals of the mapping population. The segregation ratio of Satt288 was 20 homozygotes (Minsoy alleles) : 32 heterozygotes : 16 homozygotes (T328H alleles), which fit a 1:2:1 ratio ($\chi^2 = 0.7$; $P = 0.7$) (Table 3). The recombination value between Satt288 and the *rn1* locus was calculated as 0.015 ± 0.0105 with the Linkage-1 computer program (Suiter et al. 1983).

To develop a map for the *rn1* locus, 22 additional SSR markers on MLG G were used to screen the two parental lines on a 2% (*w*/*v*) agarose gel. These were Satt131, Satt566, Satt352, Satt427, Satt594, Satt303, Satt138, Satt533, Satt564, Satt504, Satt199, Satt505, Satt400, Satt012, Satt503, Satt517, Satt612, AF162283 (annealing temperature, 53 °C), Satt472, Satt191, Sct_187, and Sat_064 (annealing temperature, 55 °C). Among these markers, Satt594, Satt138, Satt199, Satt505, Satt012, Satt612, Satt472, Satt191, and Sat_064 detected polymorphism between Minsoy and T328H. Satt199, Satt505, Satt012, Satt612, Satt472, Satt191, and Sat_064 were used with Satt288 to screen the whole mapping population and generate the map for the *rn1* locus, using MAPMAKER 2.0 (Lander et al. 1987) (Fig. 2). The *rn1* gene that conditions the necrotic root phenotype in soybean was mapped between Satt288 and Satt612 with genetic distances of 1.6 cM and 4.9 cM, respectively (Fig. 2). The segregation ratios of all markers fit a ratio of 1 homozygote (Minsoy alleles) : 2 heterozygotes : 1 homozygote (T328H alleles) (Table 3).

Fig. 1. Results of bulked segregant analysis. 1, Minsoy (*Rn1 Rn1*); 2, T328H (*Rn1 rn1*); 3, B1 (*Rn1 Rn1*); 4, B2 (*rn1 rn1*). Satt288 on molecular linkage group G detected obvious polymorphism between the two bulks and between the two parental lines. B1 (3) had the same band pattern as Minsoy (1) and B2 (4) had the same band pattern as T328H (2).

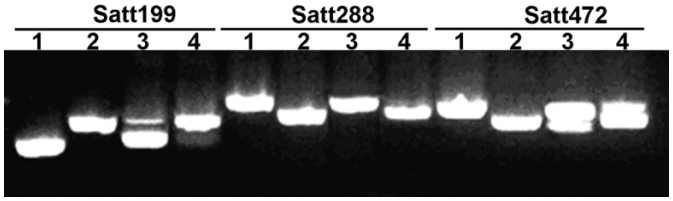


Table 3. Segregation of the *rn1* gene and SSR markers linked with the *rn1* gene in the F_2 mapping population derived from a cross of Minsoy × T328H.

	Number of F_2 plants					
Marker	A	H	B	Total	$\chi^2_{(1:2:1)}$	*P*
rn1	20	32	16	68	0.70	0.70
Satt288	20	32	16	68	0.70	0.70
Satt472	19	32	17	68	0.84	0.66
Satt199	20	36	12	68	0.35	0.84
Satt012	21	34	13	68	0.39	0.82
Satt505	20	36	12	68	0.35	0.84
Satt612	18	34	16	68	0.94	0.62
Sat_064	18	30	20	68	0.59	0.74
Expected no.	17	34	17	68		

Note: A, homozygous Minsoy genotype; H, heterozygous genotype; B, homozygous T328H genotype.

Discussion

Generation of mutants

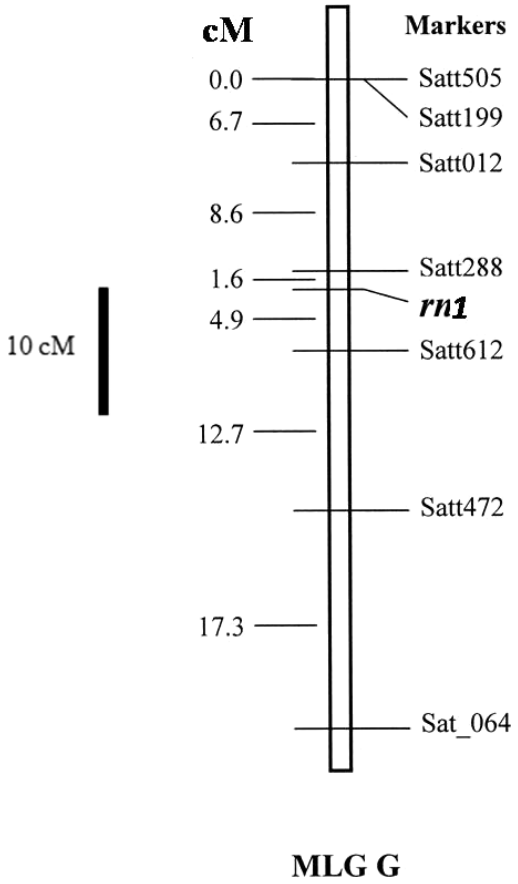
Molecular mapping studies showed that some loci were allelic (tan-saddle seed coat [*k2*], chlorophyll-deficient leaves [*y20*], and malate dehydrogenase null [*Mdh1-n*]) and were tightly linked on MLG H (Xu and Palmer 2005*b*). The male-sterile, female-sterile locus (*st8*, T352) was located on MLG J (Kato and Palmer 2003). The female partial-sterile 1 locus (*Fsp2*, T364), female partial-sterile 2 locus (*Fsp3*, T365), female partial-sterile 3 locus (*Fsp4*, T366), and female partial-sterile 4 locus (*Fsp5*, T367) were located on MLGs C2, A2, F, and G, respectively (Kato and Palmer 2004). The *w4* locus (*w4-m*) was located at one end of MLG D2 (Xu and Palmer 2005*a*). Thus, the mutants generated in the *w4* mutable line were distributed throughout the soybean genome.

The molecular mechanism(s) that generated these high-frequency mutations among progenies of germinal revertants of *w4* is not known. Genetic studies, particularly in maize and snapdragon, have shown that genes with a transposon insertion are hotspots for secondary mutations owing to imprecise excision of the transposon (Martin et al. 1988; Robertson et al. 1994). For example, the base changes may be additions (duplications) or deletions in the host sequence. The transposon also may jump into adjacent regions and transpose back to the target gene to generate new types of mutant alleles for the target gene. This may explain the origin of dilute purple flower (T321, *w4-dp*) (Palmer and Groose 1993) and pale flower (T369, *w4-p*) (Xu and Palmer 2005*a*). Alternatively, the transposon may jump into other chromosomes (Walbot 2000).

Allelism tests

The results of the allelism tests in the F_1 and F_2 generations showed that all the necrotic root mutants generated from the second gene tagging study with *w4-m* were allelic. Furthermore, these necrotic root mutants were allelic to the 7 previously identified necrotic root mutants. A total of 27 mutants (T328, T329, T330, T333, and the 23 mutants in Table 2) were identified among progeny of germinal revertants of *w4-m* (T322). Necrotic root mutant NR-4 was identified in the F_2 of a cross between Clark-*k2* and T323. T323 had been identified among progeny of a germinal revertant plant of *w4-m* (Palmer and Mahama 2005). Necrotic root mutant NR-5 was found as a spontaneous mutant in the F_2 of a cross of CLG 8 mutants with KS172-11-3, a homozygous chromosome interchange genetic stock (Palmer and Mahama 2005). It is not known whether the KS172-11-3 genetic stock would have the necrotic root *Rn1* locus on its interchanged chromosomes. The EMS-95 necrotic root mutant (T332) was identified among progeny of EMS-treated cultivar AgriPro 1776 (Palmer and Wubben 1998).

Fig. 2. SSR map for the *rn1* locus that conditions root necrosis in soybean. The *rn1* locus was mapped between Satt288 and Satt612 on molecular linkage group (MLG) G with genetic distances of 1.6 cM and 4.9 cM, respectively.



Molecular mapping

In the molecular mapping study, the *Rn1* locus was mapped to MLG G, with distances of 1.6 cM between Satt288 and *Rn1* and 4.9 cM between Satt612 and *Rn1*. The female partial-sterile mutant allele (*fsp5*, T367) and the necrotic root mutant allele (*rn1*, e.g., T328 (Ames1)) are both on MLG G, about 30 cM from each other. This is the first example of two mutant loci generated from germinal revertants of *w4-m* that map to the same chromosome, except for dilute purple flower (*w4-dp*) and pale flower (*w4-p*), which are alleles of *w4*.

Frequency and timing of reversion

The *w4-m* allele reverts at a high frequency from the recessive form to a stable dominant form. Revertants occur

both early and late during the development of the germ line. Groose et al. (1990) reported that approximately 6% of mutable alleles revert to wild type from one generation to the next.

Somatic analysis of mutable plants (flowers) revealed that reversion of *w4-m* can occur at various times during development. Early and late reversions produce, respectively, large and small revertant sectors. Both early and late reversions of *w4-m* in the development of the germ line were demonstrated by somatic and genetic analyses of clonal sectors of different phenotypes on mutable plants (flowers) (Groose et al. 1990). Reciprocal crosses of *w4-m* plants with a Harosoy-w_4 isoline produced some wild-type F_1 progeny. This indicated that late germinal reversion can occur in either male or female reproductive organs.

Preferential insertion or target specificity

The data on necrotic root phenotypes show that ASR-6 and ASR-10 generated the highest percentages and the largest numbers of necrotic root phenotypes. Both ASR-6 and ASR-10 are in the high-frequency mutable category, but ASR-6 is characterized by early excisions and ASR-10 by late excisions (Table 1). ASR-3, ASR-7, ASR-8, and ASR-9 are in the low-frequency mutable categories and few or no necrotic root phenotypes were found.

For comparison, data are presented for the percentage and number of male-sterile, female-sterile phenotypes for the same ASR families (Table 1). The sterility data will be published separately. The similarities are that no, or few, necrotic root or sterility phenotypes were evident in the low-frequency mutable categories, which comprised 2238 families of about 50 plants each. Interestingly, all 23 necrotic root mutants characterized in this study are allelic, as are all the necrotic root mutants reported in soybean from spontaneous mutations and EMS treatment. Of the 36 male-sterile, female-sterile mutants generated from the second gene tagging study, 35 are allelic at *st8* (R.G. Palmer, unpublished data); the exception is the sterility mutant from ASR-7. One of the 36 sterility mutants has been characterized genetically and is nonallelic to known male-sterile, female-sterile mutants; it was designated *st8 st8* and assigned Soybean Genetic Type Collection number T352 (Palmer and Horner 2000). The *st8* locus was located on MLG J (Kato and Palmer 2003).

For the generation of sterility mutants, late excision events seem to be a prerequisite, irrespective of low or high frequency of excision. Conversely, the generation of necrotic root mutants was apparent only at a high frequency of excision, irrespective of whether the excisions were early or late in flower petal ontogeny. The chronology of flower and ovule development in soybean has been summarized by Carlson and Lersten (2004). About 25 days before flowering, the initiation of floral primordia in leaf axil bracts begins. Next is sepal differentiation, followed by petal, stamen, and carpel initiation. About 10–14 days before flowering, ovule initiation begins, megasporocytes mature, and meiosis occurs, and then 4 megaspores are present. About 7–10 days before flowering, anther initiation begins, male archesporial cells differentiate, and then meiosis and microsporogenesis occur.

Groose et al. (1988) used periclinal chimeras of *w4-m* and showed that the germ line was of subepidermal origin and was cell layer LII. Thus, the phenotypes of flowers on *w4-m w4-m* plants can be used as an indicator of germ line reversions. This was the rationale used in both gene tagging studies, which generated large numbers of mutants.

The timing of Mutator activity of *Mu* elements in maize was tested by Robertson (1980, 1981, 1985). Ear maps of *Mu*-carrying plants had sectors of seeds (phenotypes) that produced plants with phenotypically similar allelic mutants. The conclusion was that *Mu* excision did not occur throughout plant ontogeny, but seemed to be restricted to a time shortly before and (or) during meiosis.

Robertson et al. (1988) used 3 maize Mutator-induced *a1* mutant alleles in experiments to test for a relationship between somatic activity and germinal activity. They used light mutable pattern, medium mutable pattern, and heavy mutable pattern phenotypes of aleurone tissue. Germinal activity was determined by the frequency with which an *a1* mutant (somatic tissue) transmitted new mutations to its outcrossed progeny plants. These studies showed that somatic mutability, as measured at the *a1* locus, was not a reliable predictor of germinal activity (Robertson et al. 1988).

Molecular characterization of target specificity or preferential insertion at the soybean necrotic root locus (*Rn*) has not been done. Similarly, no molecular studies of preferential insertion at the male-sterile, female-sterile locus (*st8*) have been reported. There are, however, examples from the maize literature of target specificity or preferential insertion. For example, *Ac* elements preferentially insert at locations linked to the initial (donor) element site (reviewed in Federoff 1989).

Mu elements are known to have target specificity or preferential insertion, perhaps because of a specific sequence found in the gene or a difference in the chromatin structure surrounding the gene. Do particular classes of *Mu* elements insert into different genes at equivalent frequencies? Hardeman and Chandler (1993) studied the *bz1* and *sh1* genes in maize, which are located 2 map units apart on chromosome 9, and found that each of the genes was preferentially targeted by a different class of *Mu* elements, even when the two genes were mutated in the same plant. Cresse et al. (1995) showed that *Mu1* elements inserted primarily (10-fold preferences) into regions of the maize genome that are of low copy number. They could not detect *Mu* element insertion into any specific secondary structure(s). Dietrich et al. (2002) analyzed 79 independently isolated *Mu*-induced allelic mutations at the *gl8* locus in maize. A large percentage (78%) of the insertions were located in the 5′ untranslocated region of the *gl8* gene. Thus, sequences flanking the target-site duplication seem to be involved in *Mu* insertion-site preferences.

In addition to the 24 necrotic root mutations and the 36 male-sterile, female-fertile mutations, 31 chlorophyll-deficient, tan-saddle seedcoat, malate dehydrogenase 1 mutations have been reported for this chromosome region on MLG H (Xu and Palmer 2005*b*). All *Mdh1-n y20* mutants studied were missing a 5.5 kb *Eco*RI band that corresponded to the soybean *Mdh1* gene (Imsande et al. 2001). Many of the chlorophyll-deficient (*y20*) mutants are in the same genetic background but have slightly different phenotypes (Palmer et al. 1989). This has been reported in

maize, where the phenotypes of the "same" *Mu*-induced mutations can be quite different, either from gene to gene or between alleles of the same gene (Bennetzen et al. 1993). Also in maize, transposons such as *Ac/Ds*, *En/Spm*, and *Mu* preferentially insert into relatively hypomethylated, low-copy-number DNA (Kunze and Weil 2002).

In summary, the 24 soybean necrotic root mutations induced by *w4-m* were independently derived in a gene tagging study. They are phenotypically similar and allelic, but they have not been characterized for target specificity or preferential insertion. Similarly, the sterility mutants and the chlorophyll-deficient, tan-saddle seedcoat, malate dehydrogenase 1 mutants would be ideal genetic materials for molecular characterization of target specificity or preferential transposon insertion in soybean.

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